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Differential expression of PKCα and PKCβ isozymes in CD4$^+$, CD8$^+$ and CD4$^+$/CD8$^+$ double positive human T cells

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Abstract

Using specific monoclonal and polyclonal antibodies, we have analyzed protein kinase C α and β isozyme expression in human T cells from peripheral blood (PB) and from thymus While the PKCβ isozyme was present in all T cell sub-types isolated from both PB and thymus, the α isozyme was found only in single positive CD4$^+$ thymocytes, in PB-CD4$^+$ lymphocytes and in PB-CD8$^+$ T cells from several donors It was absent from both, CD8$^+$and double positive CD4$^+$/CD8$^+$ thymocytes These results show that PKCα and -β are differentially regulated during intra-thymic development and suggest that PKCα plays a specific role in helper T cell function

Key words Protein kinase C isozyme, Human thymocyte, T lymphocyte development

1. Introduction

Protein kinase C (PKC) comprises a multigene family of protein kinases involved in a variety of cellular responses to extra cellular stimuli, including cell proliferation. In T lymphocytes PKC can be activated as a result of triggering of the T cell receptor (TcR) by antigen or by specific antibodies [1,2] Activation of the TcR induces hydrolysis of membrane-bound phospholipids, such as phosphoinositides, and yields diacylglycerol (DAG) [3]. DAG allosterically activates PKC family proteins Activation of PKCs can be mimicked by the tumor promoting phorbol ester PMA [4]

The protein products of various PKC genes are structurally and probably functionally, different. Three groups of PKC isozymes can be distinguished on the basis of their dependence on intracellular [Ca^{2+}] and their allosteric activation by phospholipid hydrolysis subproducts (for a recent review see [5]) The firstly identified group of PKCs, called classical PKCs, is composed of α, β (I and II), and γ, which contain a Ca^{2+} binding domain, and their enzymatic activity requires Ca^{2+} concentration in the micro molar range in the absence of DAG. A second group is composed of so called 'new' PKCs, (δ, ε, θ, η), which do not contain a Ca^{2+} binding domain. The third group contains of the λ, and the ζ molecules and differs from the two other groups by the absence of one of the cystein-rich domains, which prevents them from being activated by phorbol esters and probably, even if this is not yet clear, by DAG. PKC isozymes also differ by their tissue and intracellular localization although a single cell can produce several different PKC isozymes

In T cells, several previous studies demonstrated the presence of multiple PKC isozymes [6–13] In spite of increasing interest in this field, the role of discrete PKC isozymes in T cell signalling remains unclear NF-κB is one of the transcription regulators which can be activated in T cells by PMA [14]. We have reported previously, that in freshly extracted human thymocytes, activation of NF-κB by PMA did not require Ca^{2+} influx This result contrasted with results we obtained in the mature CD4$^+$ T cell line Jurkat and in non tumour T cells from peripheral blood [15] We hypothesised that this difference in Ca^{2+} requirement could be due to a difference between PKC isozyme expression in mature T cells and in thymocytes In the present study we compare the expression of classical PKC isozymes in phenotypically distinct T cell sub-types from both, thymocytes, and peripheral blood, using purified normal human T cell subpopulations, and monoclonal antibodies specific for α and β PKC isozymes Our results indicate that both PKCα and PKCβ isozymes are differentially regulated during T cell development

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Abbreviations PB, peripheral blood, PBL-T, peripheral blood T lymphocytes, PMA, phorbol 12-myristate 13-acetate, PKC, protein kinase C, DAG, diacylglycerol, NF-κB, nuclear factor κB, FCS, fetal calf serum, PHA, phytohaemagglutinin

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2. Materials and methods

2.1 Cells

Human thymi were obtained from patients undergoing cardiovascular surgery. Thymocytes were prepared as described previously [15]. Contamination of thymocytes by B cells and monocytes was analyzed by flow cytometry, using respectively, the B4-FITC and MY4-FITC antibodies (Coulter). The cells were systematically analyzed by flow cytometry using fluorescent antibodies specific for CD3, CD4, CD8 (Becton Dickinson) CD1a (Coulter) and CD25 (Immunotech) prior to protein extraction, or further treatment. Flow cytometry analysis of the phenotype of thymocytes extracted from 37 individual thymi are summarised in Table 1. The single positive CD4 and CD8 cells were purified by negative selection using magnetic adsorption on beads bound to specific antibodies for human CD4 or CD8 (Dynal). The double positive CD4/CD8 cells were obtained by positive selection using magnetic beads carrying CD1a specific antibody (kindly provided by Laurence Boumsell). The negatively selected single CD4- or CD8-positive cells were used, either immediately for protein extraction, or were cultured in RPMI 1640 supplemented with 10% FCS, standard concentrations of glutamine and antibiotics, IL2 (20 U/ml) and PHA (1 μg/ml). After 3 days of culture, a PHA-free medium was used. Unselected thymocytes were cultured in an identical manner as selected CD4 or CD8 cells and their phenotypes were assessed by flow cytometry at different periods of time. Peripheral blood T cells (PBL-T) were isolated from healthy donor's blood after depletion of erythrocytes by Ficoll/Hypaque followed by treatment with a buffer containing 155 mM NH_4Cl, 100 mM EDTA, 10 mM $KHCO_3$, pH 7.4. Monocytes and B cells were removed by adhesion on plastic dishes and nylon wool. The resulting highly enriched T cell population was phenotyped by flow cytometry.

2.2 PKC isoform determination

Protein kinase C isozymes were identified by Western blotting. Proteins from cytosol or particular fractions were isolated as described [16] and resolved on a 10% polyacrylamide gel by electrophoresis, accordingly to Porzio and Parson [17]. The transfer onto PVDF membrane (Millipore) was performed in a semi-dry Hoeffer blotter. The transfer efficiency was assessed by Ponceau Red staining. The membrane was then saturated with 3% gelatine and the blotting performed with immunoglobulin was as described [16] using the following PKC isozyme specific antisera: PKCα monoclonal antibody from Amersham (1/500 dilution), PKCβ monoclonal antibody, which does not discriminate between βI and βII forms, from Seikagaku, Japan (1/1000 dilution), polyclonal antibodies for PKCβI and PKCβII from Santa Cruz (1/2000 dilution). The antibody–antigen complexes were revealed using enhanced chemiluminescence (Amersham ECL system).

3. Results and discussion

3.1 PKC α and β in freshly extracted human thymocytes

We compared the expression of Ca-dependent PKC isozymes α, β, and γ in the Jurkat cell line, in PBL-T cells and in freshly extracted human thymocytes by immunoblotting using isozyme specific antibodies. The results are shown in Fig. 1A and B. PKCα was detectable only in Jurkat cells and PBL-T cells but not in thymocytes (Fig. 1A). As expected, PKCα was located mostly in the cytosolic compartment of Jurkat and PBL-T cells and was found in the particulate fraction after treatment with PMA. In contrast, PKCβ was found to be abundant in thymocytes, in Jurkat and PBL-T cells (Fig. 1B). However, an additional difference between thymocytes and mature-phenotype T cells was observed when polyclonal antibodies specific for the PKCβI and β-II isozymes were used. Indeed, while in Jurkat and PBL-T cells both β isoforms were present in equal amounts, in thymocytes the βII form was clearly predominant (Fig. 1B). This was also true in murine thymi (not shown). Furthermore, thymocytes and PBL-T cells contained more PKCβ than the tumor cell line (see also Fig. 3C). The third classical PKC, PKCγ, was undetected in all of the T cells tested (not shown), which agrees with previous reports showing that the expression of this isozyme is restricted to cells of the central nervous system [10,13].

These results were obtained using a large number of thymi (48 thymi tested). No obvious differences were seen with sex or age of the donor child. However, in two particular cases, traces of PKCα were detected in the non-selected, freshly isolated thymocytes (see below).

3.2 PKCα expression in cultured thymocytes and in selected cell sub-populations

We then hypothesised that the reason PKCα was not detected in human thymocytes was that PKCα expression is repressed in the context of the human thymus. Cell to cell interactions, or soluble factors secreted by thymic epithelial cells or thymocytes themselves, could exert a repressive signal for PKCα expression. In order to investigate that hypothesis, we analyzed PKCα expression in in vitro cultured human thymocytes. Fig. 2 shows a typical result obtained after 0, 4 and 7 days of cell culture in medium containing IL2. PKCα was detectable after 4 days of culture with cells from several thymi and its protein level was increased with time of culture. During cell culture, however, the cell surface phenotype of thymocytes changed as well. In the example shown in the Fig. 2, the number of CD4 and CD8 single positive cells increased from 11,8 and 11,5%, respectively, to 30% and 32%, after 7 days of culture, while the number of double positive CD4/CD8 cells decreased from 75% to 37% (data not shown). It was therefore possible that the detection of PKCα in cultured thymocytes was due to an increased number of single positive CD4 and/or CD8 cells, rather than to an in vitro de-repression of the PKCα gene. To address this question, we performed extracts from selected thymocytes at days 0 and 7 of cell culture. The results of Western blots in those extracts are shown in Fig. 3A. They demonstrated that PKCα was detected in both freshly isolated, uncultured, CD4 single

Table 1
Cell surface antigens expressed in freshly extracted human thymocytes

	Phenotype					
	CD3	CD4	CD8	CD4/CD8	CD25	CD1
%	65	12	8	78	3	92,5
S D	11	6,5	4	10	2	5

The phenotype was detemined by flow cytometry using CD specific antibodies as described in section 2. The values represent means of 37 distinct preparations of human thymocytes. S D is the standard deviation.

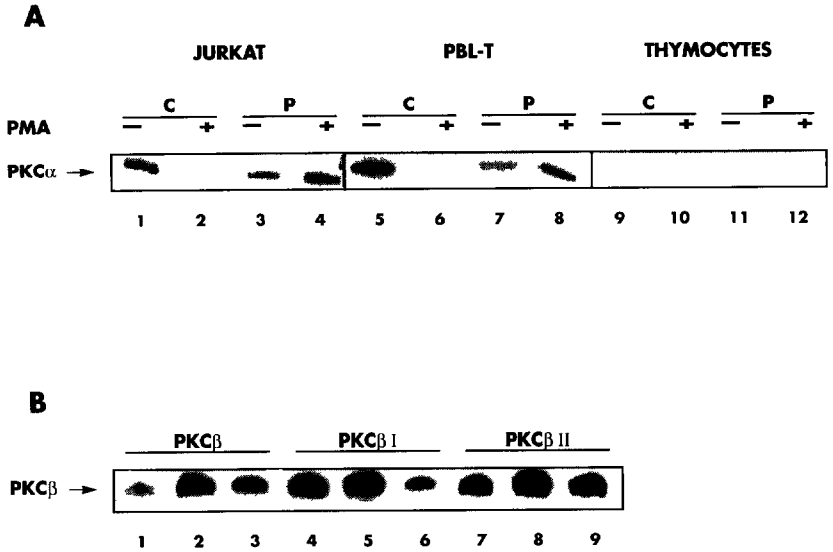

Fig 1 PKCα and -β detection in unselected T cells by Western blotting (A) Western blotting using PKCα specific monoclonal antibody with cytosolic (C) and particulate (P) extracts from Jurkat (lanes 1 to 4), PBL-T cells (lanes 5 to 8) and thymocytes (lanes 9 to 12) Equal amounts of extract were loaded (30 mg protein/ lane) from unstimulated (−), or PMA stimulated (+) cells (B) PKCβ immunoblot in Jurkat cells (lanes 1, 4, 7) PBL-T cells (lanes 2, 5, 8) and thymocytes (lanes 3, 6, 8) with monoclonal PKCβ specific antibody which recognises both βI and βII forms (lanes 1 to 3) and polyclonal antibodies specific for the PKCβI (lanes 4 to 6) and PKCβII (lanes 7 to 9)

positive thymocytes, and in cultured $CD4^+$ thymocytes (Fig 3A, lanes 3 and 7). PKCα was more abundant in cultured cells than in freshly extracted cell, suggesting that the cell culture induced over production of the enzyme PKCα was present in CD4 single positive thymocytes but was absent, or under-expressed, in double positive, and in CD8 single positive, thymocytes (Fig 3A). Thus PKCα expression was not systematically repressed by the environment of thymocytes in the thymus, since it was expressed in CD4 single positive cells but it could be correlated specifically with the acquisition of the helper CD4 single positive phenotype since it was not detected in other sub-populations of thymocytes Furthermore, these results could explain why we observed faint but detectable PKCα amounts in extracts from two unselected uncultured thymocytes. Indeed, in those particular thymi (2 out of 48 tested), CD4 single positive thymocytes represented over 28% of total cells which was far above the average content of CD4 single positive thymocytes at the day 0 of culture (see Table 1) The amount of PKCα in these unusually numerous CD4 single positive thymocytes was probably sufficient for detection by Western blotting in extracts from the total cell population

Our results indicated that $CD8^+$ thymocytes did not express detectable PKCα. This was surprising since PKCα was reported to be present in peripheral blood $CD8^+$ T cells [6] We therefore investigated the expression of PKCα at the protein level in $CD4^+$ and $CD8^+$ cells from peripheral blood of several healthy volunteers. We have found that, PKCα was expressed in $CD4^+$ T cells, but was not detected in $CD8^+$ cells from most donors (Fig 3B) However, in some donors, PKCα was detected even in $CD8^+$ PBL-T cells (Fig. 3C) In fact, the expression of PKCα in PBL-T CD8 positive cells depended on the donor considered, ranging from undetectable to amounts comparable to those found in $CD4^+$ T cells This was not the case with $CD8^+$ cells extracted from the thymus, where we never detected PKCα, and it was not the case with $CD4^+$ cells, where PKCα was systematically detected These observations tempt us to speculate that, in $CD8^+$ lymphocytes from PB, PKCα expression is not constitutive, but could be rather inducible by pharmacological, infectious or physiological events. $CD4^+$ positive T cells, in contrast, constitutively express PKCα isozyme, as soon as they mature into the single positive phenotype in the thymus. In addition, a marked difference was noticeable between the tumor cell line Jurkat and $CD4^+$ non tumor T cells from PB and thymus Indeed, lower amounts of PKCα were detected in thymocytes and PBL-T cells than in Jurkat cells (Fig. 3A,C) Moreover the ratio between PKCα and -β was in favour of PKCα in Jurkat cells, whereas in the non tumor $CD4^+$ cells, the PKCβ was clearly the predominant isozyme In a previous report, Lucas et al. [10] showed that PKCα was also more abundant in two tumor cell lines (HPB-ALL and J6) as compared to PBL-T cells, although they

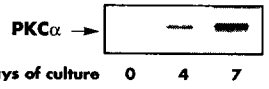

Fig 2 Immunoblot of PKCα in thymocytes cultured for 0, 4 and 7 days in RPMI containing IL2 and PHA 30 μg of protein from cytosols of unselected thymocytes were loaded onto each lane

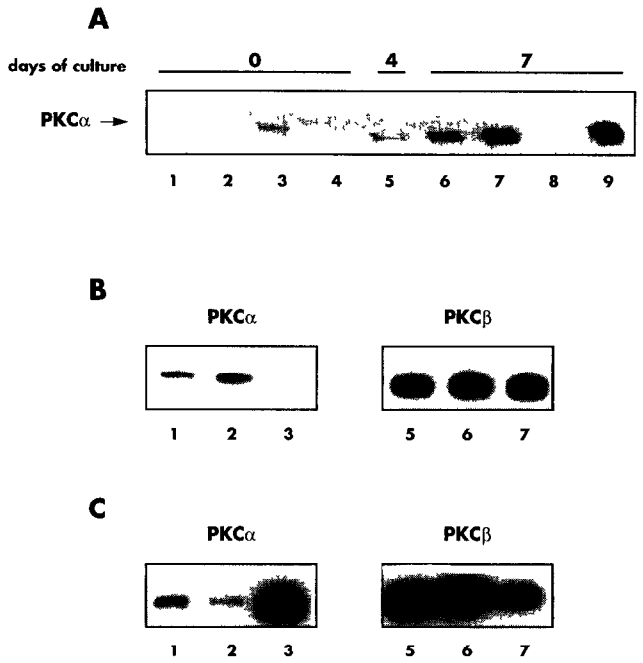

Fig 3 Immunoblots of PKCα and -β in selected populations of thymocytes and PBL-T cells (A) PKCα in extracts from uncultured (lanes 1 to 4), 4 days cultured (lane 5), and 7 day cultured (lanes 6 to 8) thymocytes and in Jurkat cells (lane 9) Lanes 1, 5 and 6, unselected thymocytes, lane 2, $CD4^+/CD8^+$ thymocytes, lanes 3 and 7, $CD4^+$, lanes 4 and 8, $CD8^+$ cells (B) Immunoblot of PKCα (lanes 1 to 3) and -β (lanes 4 to 6) in CD4 and CD8 selected PBL-T cells Lanes 1 and 4, unselected T cells, lanes 2 and 5, $CD4^+$ cells, lanes 3 and 6, $CD8^+$ cells (C) Immunoblot of PKCα (lanes 1 to 3) and -β (lanes 3 to 6) in selected $CD4^+$ (lanes 1 and 4), $CD8^+$ (lanes 2 and 6), PBL-T cells and in Jurkat cells (lanes 3 and 7) Equal amount proteins, extracted from cytosol, were loaded onto each lane

were CD4/CD8 double positive Thus, excessive production of PKCα could be part of a process involved in T cell tumorigenicity

In vitro studies of classical PKC isozymes demonstrated that PKCα is dependent on Ca^{2+}, even in the presence of molecules mimicking DAG (phosphatidylserine plus diolein) In contrast, PKCβ isozymes are almost fully active in a Ca^{2+}-free medium [13]. If this enzymatic behaviour is also true in situ, increase of $(Ca^{2+})_i$ should not affect significantly the activation of PKCβ but should change signalling through PKCα Thus our previous finding, which showed that activation of NF-κB by PMA is independent of Ca^{2+}in freshly prepared, unselected thymocytes, is consistent with the fact, that the vast majority of thymocytes produce PKCβ, but not PKCα. However, it is not clear yet, why NF-κB activation becomes Ca^{2+} dependent in cells such as Jurkat or PBL-T cells, which do express PKCα, but also PKCβ

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